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(54) Title: IN VITRO ASSAY TO DETECT INHIBITORS OF PROTEIN AND/OR mRNA BIOSYNTHESIS

(57) Abstract

A method of detecting compounds having activity as inhibitors of protein and/or mRNA biosynthesis, which method comprises incubating a mixture of reagents suitable for the synthesis of a functional reporter enzyme at a detectable rate in vitro, both alone as a control and in the presence of a test compound, detecting the functional reporter enzyme produced in the control and test mixtures and comparing the results.

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IN VITRO ASSAY TO DETECT INHIBITORS OF PROTEIN AND/OR MRNA BIOSYNTHESIS

The present invention relates to an in vitro assay for detecting inhibitors of protein and/or mRNA biosynthesis, to the use of this assay in the discovery of novel antibiotics and herbicides and in determining their mode of action and to biologically active inhibitors of protein, particularly bacterial protein, and/or mRNA biosynthesis and herbicides obtained thereby.

A non-radiochemical method for studying, in vitro, the transcription and translation of DNA was first described by G. Zubay in, for example, Ann. Rev. Genet., (1973), 7, 267-287. Modifications to this method were later described by J. Collins in Gene, (1979), 6, 29-42.

These methods were employed in research for study purposes.

Inhibition of protein and/or mRNA biosynthesis is an interesting biological effect which can have potential applications in, for example, the antibiotic and, as the applicants have found, the agrochemical and in particular the herbicidal fields. However screens employed for detecting inhibition of protein biosynthesis are low throughput and radiometric and hence unsuitable for the large scale screening of compounds necessary to discover useful biological activity or for detecting leads in these areas.

The applicants have developed a non-radiometric screen for the high throughput screening of inhibitors of protein and/or mRNA biosynthesis.

According to one aspect of the present invention there is provided a method of detecting compounds having activity as inhibitors of protein and/or mRNA biosynthesis, which method comprises incubating a mixture of reagents suitable for the DNA-directed synthesis in vitro of a functional reporter enzyme at a detectable rate, both alone as a control and in the presence of a test compound, detecting the functional reporter enzyme produced in the control and test mixtures and comparing the results.

The test is suitably carried out on a microtitre scale.

Conditions such as the incubation period for protein synthesis, time of assay, temperature and reagent concentrations etc., can be adapted to ensure that detectable quantities of reporter enzyme are generated in the control test within these time periods most convenient for high throughput screening. For instance, an incubation period for protein synthesis is suitably less than about 2 hours and preferably about 1 hour.

Suitable reporter enzymes are those which can be assayed for example by addition of chromogenic or fluorogenic substrates. These will include β-galactosidase and β-glucuronidase. Alternatively firefly luciferase could be used to generate light.

The mode of detection of the reporter enzyme will depend upon the particular enzyme chosen. For example colorimetric or light generation assay techniques can be employed depending upon the enzyme. Assay chemicals are added either intially to the reaction mixture and/or at suitable points during the method.

A preferred reaction for use in the method of the invention is based upon that described by G. Zubay supra and further modified by J. Collins supra. A mixture of reagents suitable for enzyme synthesis comprises the following components:

1) an amino acid mixture;
2) a plasmid DNA encoding the reporter enzyme;
3) an S30 extract from a prokaryotic organism lacking the said reporter enzyme activity; and
4) a low molecular weight compound mix comprising the reagents required for protein synthesis in vitro; and
5) magnesium ion.

The amino acid mixture is suitably a mixture of the 20 protein L-amino acids in equivalent proportions. However, the concentrations of individual amino acids in the mixture can be varied (for example, to investigate whether an inhibitor is likely to act by inhibiting any of the aminoacyl-tRNA synthetases). In such experiments, the concentrations of individual amino acids are varied and it is observed whether there is any antidoting effect through increasing the concentration of a specific amino acid. This can be taken to indicate that the particular inhibitor is most likely to be acting as a competitive inhibitor of the corresponding aminoacyl-tRNA synthetase.

The concentration of amino acids is varied suitably from 0.01 to 10mM. We have found that a reduction of the concentration of amino acid mixture relative to that described by G. Zubay supra is preferred in order to maximise the sensitivity of the test for the detection of protein biosynthesis inhibitors and, in particular, inhibitors of the aminoacyl-tRNA synthetases.

The plasmid DNA is suitably any convenient plasmid DNA carrying the lac Z gene, such as that commercially available from Promega.

Suitable S30 extracts are similar to those described by G. Zubay supra, and may include commercially available extracts such as those sold by Promega. A method of preparing such an extract is exemplified hereinafter.

The low molecular weight compound mix can contain nucleosides, salts, buffers, solvents etc., as would be apparent to the biochemist. An example of a suitable low molecular weight compound mix is given hereinafter.

The optimum amount of magnesium ion for the protein synthesising reaction needs to be established and standardised for each new batch of E. coli or S30 extract and of low molecular weight compound mix which is prepared. (Having established the amount of magnesium ion necessary to produce the most β-D-galactosidase in initial controls, and which is used in a preferred embodiment of the present invention, then this amount is used for all subsequent inhibitor testing until either all of the S30 extract or the low molecular weight component mix is used up). A convenient source of magnesium ion is magnesium acetate. In practice, the optimum amount is usually found somewhere between 5 and 30 mM of added magnesium acetate (i.e. concentration added to the protein synthesising reaction).

In a preferred embodiment, the present invention provides a colorimetric in vitro method for detecting inhibitors of transcription (RNA polymerase) and translation (protein synthesis) in an E. coli extract, which method comprises supplementing the E. coli extract with tRNA and cofactors required for biosynthesis, priming the mixture with plasmid DNA carrying the lac Z gene, allowing the primed mixture to synthesize β-D-galactosidase, quantitatively detecting the amount of enzyme made, and determining whether the presence of a potential inhibitor has reduced the β-D-galactosidase activity relative to controls.

The E. coli strain chosen for making the extract is one which lacks β-D-galactosidase (e.g. the readily available lac deleted strain MC1061). The cofactors include the amino acid mixture and the low molecular weight compound mix comprising the basic chemicals required for protein synthesis in vitro as described above.

The β-D-galactosidase is suitably detected and quantitated by adding a chromogenic substrate such as o-nitrophenyl β-D-galactoside. Inhibitors of

protein synthesis are detected by measuring the reduction in yellow colour absorbing at 410 nm formed in microtitre wells which contain inhibitor. Control experiments using β-D-galactosidase (commercially available from Sigma (UK) Ltd) are then necessary to distinguish between genuine inhibitors of mRNA or protein synthesis and molecules which are merely inhibitors of β-D-galactosidase itself.

Inhibitors of bacterial RNA polymerase and/or protein biosynthesis include many antibiotics and, because of the close similarity between the protein synthesis machinery of chloroplasts and of bacteria, also herbicides. The assay therefore provides a novel means of screening for compounds potentially useful as herbicides or antibiotics or as a means of providing in vitro active leads around which a programme of analogue synthesis could then lead to compounds useful as antibiotics or herbicides.

Thus, according to another aspect of the present invention, there is provided a method of screening for compounds potentially useful herbicides, which method comprises incubating a mixture of reagents suitable for the synthesis of a functional reporter enzyme at a detectable rate in vitro, both alone as a control, and in the presence of a test compound, detecting the reporter enzyme produced in the control and test mixtures and comparing the results.

The assay also useful as an indicator of herbicide and/or antibiotic mode of action. The test will indicate whether a lead with an unknown mode of action is or is not an inhibitor of bacterial protein biosynthesis. In many cases, it will be possible to use the test to further narrow down the site of action to a more specific site within transcription/translation. In particular, it is possible to diagnose whether compounds act as specific inhibitors of any one of the aminoacyl-tRNA synthetases. Inhibitors of these are particularly valuable having use as both antibiotics and herbicides such as those described in copending International Patent Publication No. WO93/19599. So, for example, competitive inhibitors of isoleucyl-tRNA synthetase can be readily identified through the specific ability of isoleucine to antagonise the inhibition observed in this test.

Further according to the present invention there is provided a biologically active inhibitor of bacterial protein and/or mRNA biosynthesis (the transcription and translation of a functional protein gene product)

identified using the method according to the present invention.

The invention also comprises herbicidal compounds consisting of, or derived from such inhibitors, but excluding those of International Patent Publication No. WO93/19599 of general formula (I) or (IA) or (IB) where Y represents a group of sub-formula (IC) or (ID or (IE) and wherein R^2 is a group $CO\text{-}XR^3$ wherein X is O or S and R^3 is hydrogen or an agrochemically acceptable ester-forming radical; or R^2 is a group $-R^4$ wherein R^4 is an optionally substituted aryl or heterocyclic group; or R^2 is a group $CO\text{-}NR^5R^6$ wherein R^5 and R^6 are the same or different and each represent an agrochemically acceptable amide-forming radical; stereoisomers of the compounds of formula (I), (IA) and (IB) and salts of the compound of formula (I), (IA) and (IB) wherein R^2 is $COXR^3$, X is O and R^3 is hydrogen.

The following Examples illustrate the method of the present invention.

EXAMPLE 1

The following were prepared:

1. Low molecular weight compound mix (reagents generally of the purest grades available)-

Dissolve Tris (tris(hydroxymethyl)aminomethane) in H_2O and mix the following reagents in the order listed below.

440mM	Tris buffer
13.7mM	DL-dithiothreitol
9.50mM	Adenosine 5'-triphosphate (disodium salt from Equine muscle)
5.50mM	Cytideine 5'-triphosphate (tris salt, type VI)
5.50mM	Guanosine 5'-triphosphate (tris salt, type VI)
5.50mM	Uridine 5'-triphosphate (tris salt, type VI)
0.21M	Phosphoenolpyruvate (mono(cyclohexylammonium)salt)
0.375ml/ml	40% Polyethylene glycol 6000
0.1ml of stock/ml	Folinic acid (freshly prepared 2.7 mg/ml stock solution in water)
2.50 mM	Adenosine 3'-5'-cyclic monophosphate
62.4µl of stock/ml	Transfer ribonucleic acid (type XXI from E. coli, strain W available from Sigma (UK) Ltd, lyophilized). Freshly made up as a 17.4mg/ml stock solution in water
0.28 M	Ammonium acetate

0.56 M	Potassium acetate
19 mM	Calcium acetate

The solution was made up in the order above and finally adjusted to pH 8.2 with 5M potassium hydroxide.

2. Amino Acid Mixture

This was a mixture of the 20 protein L-amino acids at 2.5mM of each.

3. Lac-Z plasmid DNA

DNA pGem carrying the gene encoding β-D-galactosidase (commercially available from Promega)

4. Magnesium acetate

Purest grade available (stock solution approximately 0.5M).

5. S30 extract

The preparation of this is similar to that described by G. Zubay supra and is described in greater detail below.

6. Growth of bacterial cells.

The bacterial strain E. coli MC1061 was grown in a rich medium (as described by G. Zubay supra) at 28°C in fermenters run at a working volume of 14l. Samples were removed throughout the fermentation to measure the O.D. 550nm.

After 11.5 hours the cells were harvested. The culture temperature was reduced to 20°C and the first six litres harvested into pre-chilled centrifuge pots. The remaining culture was harvested into flasks and kept on ice. Centrifugation of both batches was carried out in a Sorvall GS3 6x500 rotor at 5000 rpm and 5°C for 20 minutes. Cell pellets were snap frozen in liquid nitrogen, weighed and stored at -80°C.

7. Preparation of broken cell (S30) extract.

The chemicals used were generally of ANALAR (or supplier's equivalent) quality. The buffer used throughout the following preparation is 10mM Tris-acetate pH 8.2 containing 14mM magnesium acetate, 60mM potassium acetate and 1mM DL-dithiothreitol.

20g of the E. coli cell pellets prepared above was resuspended in 500ml of buffer also containing 7mM 2-mercaptoethanol. The cell suspension was centrifuged at 12000 rpm and 4°C for 5 minutes in a Sorvall GSA 6x250 rotor. The supernatant was discarded and the above resuspension and centrifugation steps repeated. The pellet was resuspended in 1.25ml of buffer per gram of cells. The cells were lysed by one pass through a French

Pressure cell at 8000psi ($5.5x10^4$kPa). Immediately 10µl of 0.1M DTT (dithiothreitol) was added per ml of extract. The extract was centrifuged at 18000 rpm and 4°C for 30 minutes in a Sorvall 8x50 rotor. 80% of the supernatant is carefully removed and the previous centrifugation and extract removal steps repeated.

A solution of 0.29M Tris buffer containing 9.2mM magnesium acetate, 13.4mM adenosine 5'-triphosphate (disodium salt from Equine muscle), phosphoenolpyruvate (mono(cyclohexylammonium)salt), 4.4mM DL-dithiothreitol, 4.3 units of fresh pyruvate kinase (EC 2.7.1.40, Sigma type III from rabbit muscle) and 0.132 mM of each of the 20 protein L-amino acids was prepared. The solution was adjusted to pH 8.2 and then added to the above supernatant with 0.3 ml being added per ml of final supernatant. This is then left to incubate in a slow shaking water bath for 80 minutes at 37°C in the dark.

Approximately 20ml samples of the above extract are dialysed against four changes (each 1l) of cold buffer at 4°C over the course of 4 hours. The product is the S30 extract, which was then frozen and stored as beads in liquid nitrogen. Aliquots were then thawed and used as required.

8. Inhibitors

Test Compounds 1-3 and some known antibiotics were dissolved in appropriate concentrations up to 100ppm in 4% DMSO (dimethyl sulfoxide) or H_2O. Blank solutions were used as appropriate. Control experiments indicate that DMSO did not interfere at 4% in this solution (i.e. 1% in the protein synthesis reaction).

9. Substrate solution

1.82mM o-nitrophenyl β-D-galactopyranoside in 0.1M phosphate buffer (sodium salt) at pH 7.3 containing 0.1 M 2-mercaptoethanol.

8. Sodium carbonate

1.0 M sodium carbonate

Method

Using the above solutions, assays were carried out as described below.

Protein synthesising reaction

Potential inhibitors, the amino acid mixture, the S30 extract, plasmid DNA and the low molecular weight compound mix were mixed together in the well of a 96 well microtiter plate in the following proportions:

10µl low molecular weight component mix

2µl amino acid mixture
1.5-3.0 µg lac Z plasmid DNA
17µl S30 extract
10µl of inhibitor (or, in controls, 10 µl of water, or 4% DMSO in H_2O, according to what the inhibitors are dissolved in).
Magnesium acetate - appropriate amount determined as described above.

Protein synthesising reaction

Each well was made up to 42 µl volume with H_2O. A reaction was started by the final addition of the low molecular weight compound mix and mixing, or by pre-mixing all the components at ice temperature and starting the reaction by raising the temperature to 37°C. The mixture was allowed to react at room temperature for an hour.

Enzyme assay

A solution containing 200 µl o-nitrophenyl-β-D-galactoside in buffer was then added to initiate the assay of the enzyme synthesised. After 1-3 hours, 100 µl sodium carbonate solution was added and the absorbance measured, at 410 nm, referenced against 570 nm, using a Dynatech 7000 microtitre plate reader.

Results

The results are shown in Table 1. In the Table:-

i) Concentrations of compounds refer to their final concentrations in the initial protein synthesis reaction mixture.

ii) Percent inhibition is calculated with reference to control values (absorbance obtained in assays containing no inhibitor) and to blank values (background absorbance obtained in assays containing no plasmid DNA).

$$\text{Percent Inhibition} = \left\{ 1 - \frac{\text{sample value-blank value}}{\text{control value-blank value}} \right\} \times 100$$

TABLE I

Inhibitor	Concentration(µM)	% Inhibition
Chloramphenicol	48	100
Streptomycin	48	66
Compound 1	48	100
" "	1	22
" "	0.5	7

Compound 2	24	43
Compound 3	2.3	18

CONTROL EXAMPLE

The purpose of the control experiments is to check that the inhibitors discovered really are inhibitors of protein biosynthesis and not simply inhibitors of β-D-galactosidase.

When testing potential inhibitors at a typical test rate of 20-40 ppm, most of the "hits" detected are likely to be inhibitors of transcription or translation. Occasionally, apparent hits result from compounds being direct inhibitors of β-D-galactosidase. Such inhibitors can easily be detected by running the test using β-D-galactosidase enzyme purchased from Sigma (UK) Ltd (Sigma grade VI from E. coli) in place of the initial mixture used in the protein synthesis reaction described above. (The appropriate amount of diluted enzyme can readily determined by experiment as that required to produce an absorbance change at 410nm of around 0.5-1.0 over the course of an hour).

EXAMPLE 2

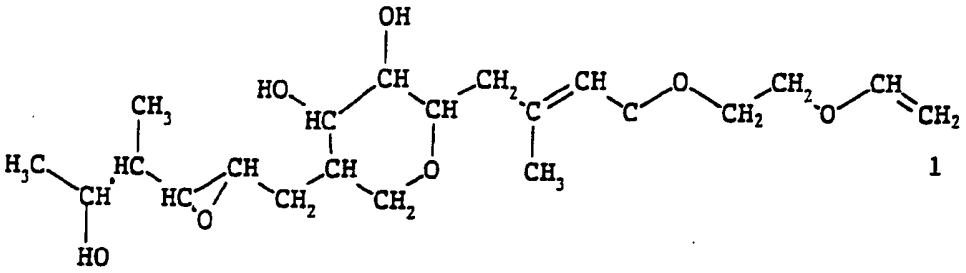
The assay may also be used as a diagnostic test to determine the exact mode of action of such inhibitors as described above. In this example the inhibition of Compound No. 2 is shown to be reversed by L-isoleucine indicating isoleucyl-tRNA synthetase as the site of inhibition.

TABLE II

Inhibitor	Concentration (μM)	% Inhibition	Isoleucine (mM)
Compound 2	24	43	0.119
	24	4	1.16

CHEMICAL FORMULAE

(IN DESCRIPTION)



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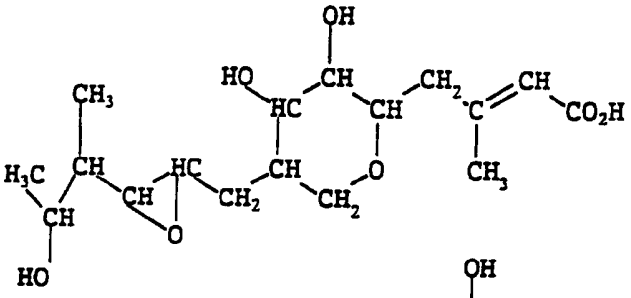

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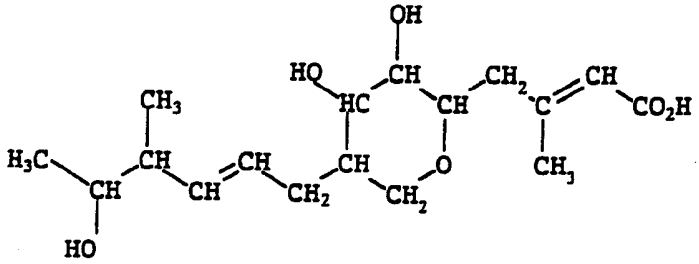

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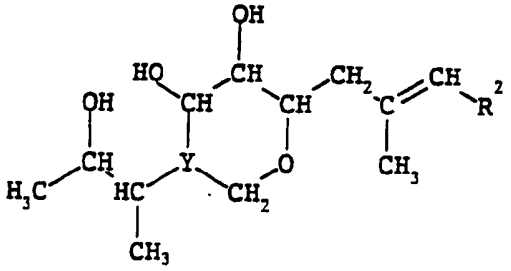

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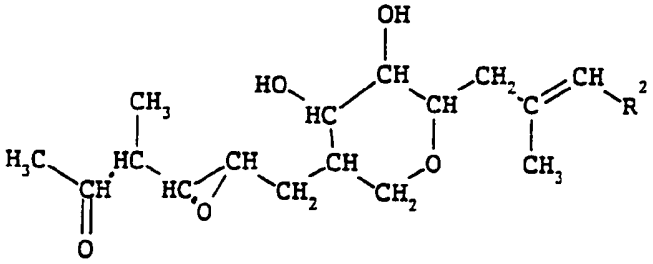

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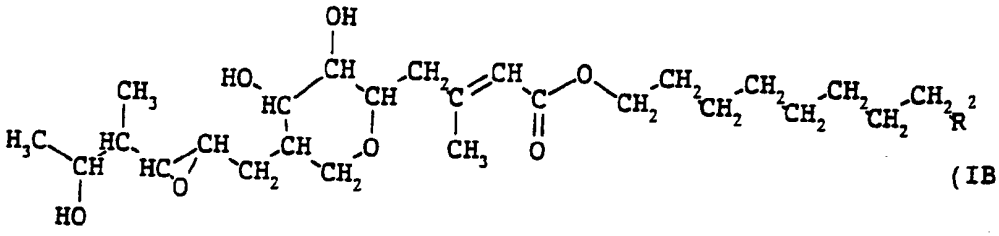

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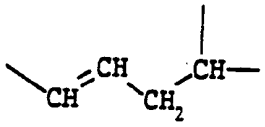

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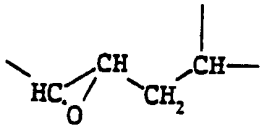

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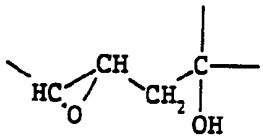

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CLAIMS

1. A method of detecting compounds having activity as inhibitors of protein and/or mRNA biosynthesis, which method comprises incubating a mixture of reagents suitable for the synthesis of a functional reporter enzyme at a detectable rate in vitro, both alone as a control and in the presence of a test compound, detecting the functional reporter enzyme produced in the control and test mixtures and comparing the results.

2. A method of screening for compounds potentially useful herbicides and/or antibiotics, which method comprises incubating a mixture of reagents suitable for the synthesis of a functional reporter enzyme at a detectable rate in vitro, both alone as a control and in the presence of a test compound, detecting the functional reporter enzyme produced in the control and test mixtures and comparing the results.

3. A method of determining the mode of action of herbicides and antibiotics, which method comprises incubating a mixture of reagents suitable for the synthesis of a functional reporter enzyme at a detectable rate in vitro, both alone as a control and in the presence of a test compound, detecting the functional reporter enzyme produced in the control and test mixtures and comparing the results.

4. A method according to any one of claims 1 to 3, in which the said mixture of reagents comprises :
   1) an amino acid mixture;
   2) a plasmid DNA encoding the functional reporter enzyme;
   3) an S30 extract from a prokaryotic organism lacking the said functional reporter enzyme activity; and
   4) a low molecular weight compound mix comprising the reagents required for protein synthesis in vitro and;
   5) magnesium ion.

5. A method according to claim 4, in which the plasmid DNA carries the lac Z gene, and the S30 extract is from an E. coli strain which lacks β-D-galactosidase.

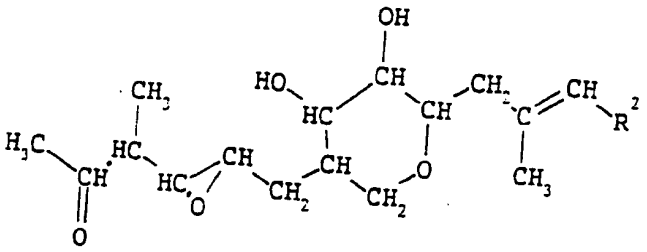
6. A method according to claim 5, in which the E. coli strain is the lac deleted strain MC1061.

7. A method according to any preceding claim, in which detection is by a colorimetric or light generation assay technique.

8. A colorimetric in vitro method for detecting inhibitors of transcription (RNA polymerase) and/or translation the synthesis of a functional protein gene product in an E. coli extract, which method comprises supplementing the E. coli extract with tRNA and cofactors required for biosynthesis, priming the mixture with plasmid DNA carrying the lac Z gene, allowing the primed mixture to synthesize β-D-galactosidase, quantitatively detecting the amount of enzyme made, and determining whether the presence of a potential inhibitor has reduced the β-D-galactosidase activity relative to controls.

9. A biologically active inhibitor of bacterial protein and/or mRNA biosynthesis (transcription and translation) identified using the method of any preceding claim.

10. A herbicidal compounds consisting of, or derived from, an inhibitor according to claim 9, but excluding those of general formula (I) or (IA) or (IB)



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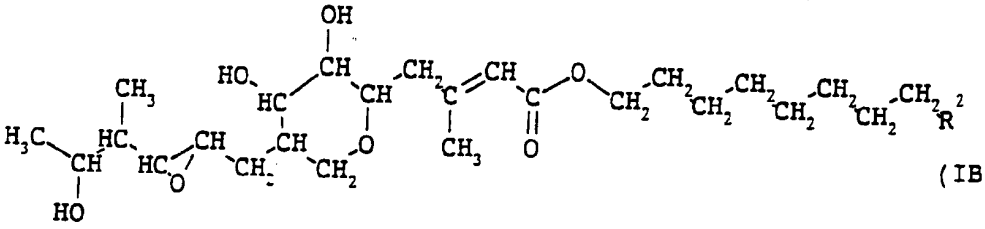

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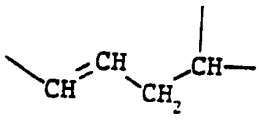
where Y represents a group of sub-formula (IC) or (ID or (IE)



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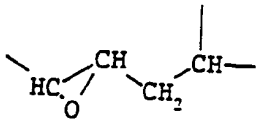

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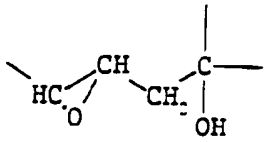

(IE)

and wherein R^2 is a group $CO\text{-}XR^3$ wherein X is O or S and R^3 is hydrogen or an agrochemically acceptable ester-forming radical; or R^2 is a group $-R^4$ wherein R^4 is an optionally substituted aryl or heterocyclic group; or R^2 is a group $CO\text{-}NR^5R^6$ wherein R^5 and R^6 are the same or different and each represent an agrochemically acceptable amide-forming radical; stereoisomers of the compounds of formula (I), (IA) and (IB) and salts of the compound of formula (I), (IA) and (IB) wherein R^2 is $COXR^3$, X is O and R^3 is hydrogen.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 94/02088

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/18 //C12Q1/25

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ANNUAL REVIEW OF GENETICS, vol.7, 1973, PALO ALTO, CA, US pages 267 - 287 G. ZUBAY 'In vitro synthesis of protein in microbial systems' cited in the application see page 278, paragraph 2 ---	1-10
A	MANIATIS ET AL. 'Molecular Cloning' 1989 , COLD SPRING HARBOR LABORATORY PRESS see page 18.81 - page 18.84 --- -/--	

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 January 1995	16.02.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Molina Galan, E

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INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/GB 94/02088

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GENE., vol.6, 1979, AMSTERDAM NL pages 29 - 42 J COLLINS 'Cell-free synthesis of proteins coding for mobilisation functions of ColE1 and transposition functions of Tn3' cited in the application ------	

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